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REMARKS

Formal Matters

Claims 26, 27, 29, 30 and 32 are pending after entry of the amendments set forth herein.

Claims 26, 27, 29, 30 and 32 were examined and rejected.

Claims 27 and 30 are amended. The amendments to the claims were made solely in the interest of expediting prosecution, and are not to be construed as an acquiescence to any objection or rejection of any claim. Support for the amendments to the claims is found in the claims as originally filed, and throughout the specification, in particular at the following exemplary locations: page 33, lines 5 and 6, and page 7 lines 8-9. Accordingly, no new matter is added.

Applicants respectfully request reconsideration of the application in view of the remarks made herein.

Sequence Listing

The Applicants note a discrepancy between the sequence listing filed in this application and the figures of the application, and wish to correct the discrepancy with a substitute Sequence Listing. Specifically, the “Z” at the C-terminus of each of the polypeptide sequences in the figures indicates that the C-terminus of each of the polypeptides and is not an encoded amino acid. This would be apparent to one of skill in the art because the “Z” is encoded by a stop codon in the polynucleotides encoding those polypeptides (i.e., the polynucleotides of Figs. 1, 3, 5, 7 and 9), and because “Z” is not part of the single letter code for the 20 naturally-occurring amino acids (see Exhibit A). Accordingly, the amino acid at the end of each of SEQ ID NOS:2, 4, 6, 8 and 10 (corresponding to the polypeptides of Figs. 2, 4, 6, 8 and 10) should be the amino acid adjacent to the C-terminal “Z”. In other words, “Z” represents no amino acid, and should not be represented as an amino acid in the sequence listing.

However, the Sequence Listing erroneously indicates that the last amino acid of each of those polypeptides (SEQ ID NOS:2, 4, 6, 8 and 10) is “Glx”. “Glx” according to the IUPAC code, is either Glutamine (Glu) or Glutamic acid (Gln). As discussed above, there should be no amino acid at this position in any of the polypeptides of the sequence listing, according to the nucleotide sequences set forth in the figures. Accordingly, the Applicants respectfully submit that the sequence listing contains errors, and, as discussed above, these errors would have been immediately recognizable as errors

because the codons encoding the “Glx” amino acids are stop codons, and, as such, encode no amino acid.

In order to correct this error, the Applicants submit herewith a substitute Sequence Listing in which the “Glx” residues at the end of each of the polypeptides in question has been deleted. No other changes have been made to the sequence listing. Applicants respectfully request that the sequence listing currently on file is substituted with that supplied herewith.

Interview Summary

The Applicants wish to express their gratitude to Examiners Ungar and Eyler for the telephone interview conducted on May 3, 2004, with Applicants' representatives James Keddie, Carol Francis and James Diehl. The outstanding rejections were discussed, as well as arguments to overcome those rejections.

Examiner Eyler indicated that the basis of all remaining rejections is the assertion that no data is shown for SEQ ID NO:8 in the instant application. The Examiners reasoned that data obtained for SEQ ID NOS:2, 4 and 6 could not be extrapolated to SEQ ID NO:8 because the Applicants did not show that any domain responsible for the described activity is also present in SEQ ID NO:8.

Examiner Eyler indicated that post-filing data relating to SEQ ID NO:8 or suitable reasoned argument would be sufficient to withdraw the rejections.

The Response in General

Polypeptides having the amino acid sequence set forth SEQ ID NOS:2, 4 and 6 possess p53-modulatory activity. This is shown in Fig. 12 of the instant application. The Applicants respectfully submit that SEQ ID NO:8 must necessarily have a p53-modulatory activity identical to that of SEQ ID NOS:2, 4 and 6. This assertion is based on the following:

- 1) The polypeptides of SEQ ID NOS:2, 4, 6 and 8 are splice variants of the same gene product. As a result, each of the polypeptides of SEQ ID NOS:2, 4, 6 and 8 share a region that is identical in amino acid sequence.
- 2) Each of the polypeptides of SEQ ID NOS:2, 4 and 6 exhibit p53-modulatory activity (see, e.g., Fig. 12 of the application).
- 3) The polypeptides of SEQ ID NO:2 and SEQ ID NOS:4 and 6 differ in overall amino acid sequence. Specifically, SEQ ID NO:2 lacks the N-terminal amino acid sequence found in

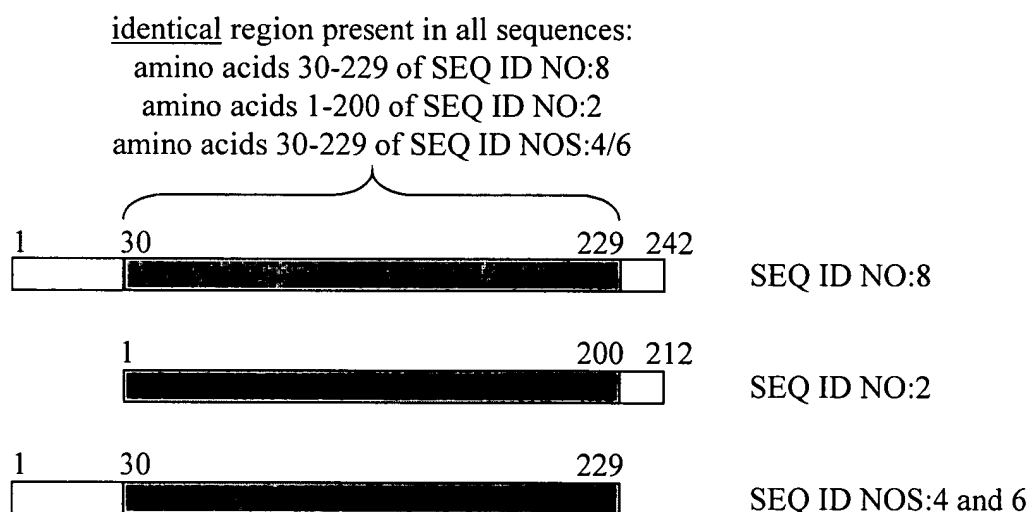
SEQ ID NOS:4 and 6; SEQ ID NOS:4 and 6 lack a C-terminal amino acid sequence found in SEQ ID NO:2. Thus the domain that confers p53-modulatory activity must be that amino acid sequence in common with SEQ ID NOS:2, 4 and 6.

- 4) The polypeptide of SEQ ID NO:8 contains that same amino acid sequence that confers p53-modulatory activity upon the polypeptides of SEQ ID NOS:2, 4 and 6.
 - a. The N-terminal amino acid sequence of SEQ ID NO:8 should not render the polypeptide nonfunctional, since this amino acid sequence is the same as the N-terminal amino acid sequence of the polypeptides of SEQ ID NOS:4 and 6, which polypeptides have p53-modulatory activity.
 - b. The C-terminal amino acid sequence of SEQ ID NO:8 should not render the polypeptide nonfunctional, since this amino acid sequence is the same as the C-terminal amino acid sequence of the polypeptides of SEQ ID NO:2, which polypeptide has p53-modulatory activity.
- 5) Apart from its N-terminal 15 amino acids, the polypeptide of SEQ ID NO:8 is identical to the amino acid sequence of a known p53 modulator, p28ING5.

Applicants' position is set forth in more detail below.

The polypeptides of SEQ ID NOS:2, 4, 6 and 8 are splice variants of the same gene product and share extensive amino acid sequence identity

SEQ ID NOS:2, 4, 6 and 8 are splice variants of the same gene product, and as a result are very related to each other in sequence. In order to show the relationship between SEQ ID NOS:2, 4, 6 and 8, a diagram schematically showing an alignment of SEQ ID NOS:2, 4, 6 and 8 is drawn below. A figure showing an alignment of the amino acid sequences of SEQ ID NO: 2, 4 and 8 is attached hereto as Exhibit B.



SEQ ID NO:8 is longer than each of SEQ ID NOS:2, 4 and 6. The N-terminal sequence from residues 1 to 29 is the same as those of SEQ ID NOS:4 and 6; the C-terminal sequence from residues 230 to 242 is the same as residues 210-212 of SEQ ID NO:2. Notably, SEQ ID NOS: 2, 4, 6 and 8 all contain an identical 200 amino acid region. Since SEQ ID NOS:2, 4 and 6 each exhibit p53-modulatory activity, then it is this shared region that confers the p53-modulatory activity of these polypeptides.

Since SEQ ID NO:8 shares extensive amino acid sequence with each of SEQ ID NOS:2, 4 and 6, including the 200 amino acid sequence shared by SEQ ID NOS:2, 4 and 6, it would be reasonable to conclude that SEQ ID NO:8 has a p53-modulatory activity of SEQ ID NOS:2, 4 and 6.

In other words, the fact that each of SEQ ID NOS:2, 4 and 6 have p53-modulatory activity indicates that neither the 29 amino acid “addition” to the N-terminus of SEQ ID NO:8, nor the 11 amino acid “addition” to the C-terminus of SEQ ID NO:8 any effect on p53-modulatory activity since these domains are present in SEQ ID NOS:4 and 6 or SEQ ID NO:2, respectively (which each possess this

p53-modulatory activity). There is no reason to expect that SEQ ID NO:8 would not have the same activity as SEQ ID NOS:2, 4 and 6 because SEQ ID NO:8 does not lack any sequence that is present in SEQ ID NOS:2, 4 or 6.

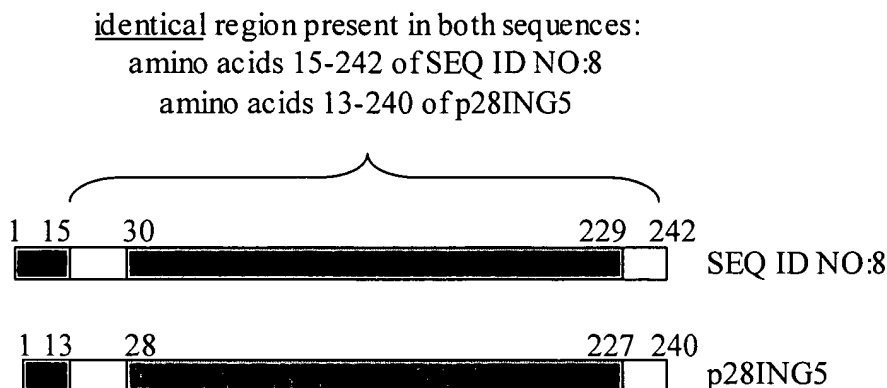
Further, because SEQ ID NOS:2, 4 and 6 have p53-modulatory activity, and these proteins only differ in their amino acid sequence at their N- and C-termini, the Applicants respectfully submit that one of ordinary skill in the art would recognize that the domain responsible for p53-modulatory activity is the shared 200 amino acid region shared by these polypeptides. Since SEQ ID NO:8 contains an identical domain (amino acids 30-229), the Applicants respectfully submit that one of skill in the art would recognize that SEQ ID NO:8 contains the domain responsible for p53-modulatory activity. Since SEQ ID NO:8 possesses this domain, and contains only additional N- and C-terminal amino acid sequence which are found in proteins having p53-modulatory activity, the polypeptide of SEQ ID NO:8 must also exhibit p53-modulatory activity.

Accordingly, the Applicants respectfully submit that one of ordinary skill in the art, in view of the results presented in Fig. 12 of the instant specification, would recognize that a polypeptide having the amino acid sequence of SEQ ID NO:8 would possess the same p53-modulatory activity as SEQ ID NOS:2, 4 and 6. Accordingly, the Applicants submit that an adequate showing of has been made to satisfy the Office's questions about the utility of SEQ ID NO:8.

Further, the Applicants respectfully submit that there is data in a peer-reviewed journal (published after the filing date of the instant application) that supports the Applicants position that a polypeptide having the sequence of SEQ ID NO:8 has p53-modulatory activity. The Applicants refer the Office to the attached Exhibit C, a paper published in *Cancer Research* entitled "p29ING4 and p28ING5 bind to p53 and enhance p53 activity". As discussed in the abstract of this paper "p29ING4 or p28ING5 overexpression resulted in a diminished colon-forming efficiency...and an induction of apoptosis in a p53 dependent manner". The authors of this paper conclude that "p29ING4 and p28ING5 may be significant modulators of p53 function". The findings of this paper therefore support a role for p29ING4 and p28ING5 in modulating p53 activity, in a very similar way to the proteins described in the instant specification.

p28ING5 is another splice variant from the same gene as SEQ ID NOS:2, 4, 6 and 8, and is more related to SEQ ID NO:8 than SEQ ID NOS:2, 4 and 6. Again, in order to show the relationship between SEQ ID NO:8 and p28ING5, a diagram schematically showing an alignment of SEQ ID NO:8 and

p28ING5 is drawn below. A figure showing an alignment of the amino acid sequences of SEQ ID NO:8 and p28ING5 is attached hereto as Exhibit D.



SEQ ID NO:8 is identical to p28ING5, except for the first 15 amino acids of SEQ ID NO:8 (corresponding to the first 13 amino acids of p28ING5). In other words, SEQ ID NO:8 and p28ING5 are identical over 227 amino acids and differ from each other only in 15 amino acids at the extreme N-terminus of the protein. These 15 amino acids are thus not essential for p53-modulatory activity of p28ING5. As the Applicants have shown, a protein that is otherwise identical to p28ING5 but does not contain these 15 amino acids (i.e., SEQ ID NO:2) possesses p53-modulatory activity.

Accordingly, the Applicants respectfully submit that the paper submitted as Exhibit C shows that a protein that is identical to SEQ ID NO:8 except for 13 amino acids at its N-terminus binds p53 and has p53-modulatory activity. Because the Applicants have shown that this 13 amino acids is not necessary for p53-modulatory activity, there is no reason why SEQ ID NO:8 should not also possess this activity.

The Applicants respectfully submit that there is overwhelming factual evidence that SEQ ID NO:8 is a modulator of p53 activity.

Pursuant to the comments made by Examiner Eyler during the afore-mentioned interview, the Applicants respectfully submit that the remaining rejections may be withdrawn.

Rejection of claims under 35 U.S.C. § 112, first paragraph (written description)

Claim 27 is rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Applicants respectfully traverse this rejection.

Without wishing to acquiesce to the correctness of this rejection, the claims have been amended to recite polypeptides that are have 95% sequence identity to SEQ ID NO:8 *and can activate a p53 binding site controlled promoter when introduced into a mammalian cell*. The Applicants respectfully submit that in view the amendment and the above general discussion, this rejection has been adequately addressed and may be withdrawn.

Rejection of claims under 35 U.S.C. § 101 (utility)

All claims are rejected under 35 U.S.C. § 101 for lacking patentable utility.

The Applicants respectfully submit that this rejection has been adequately addressed in the general discussion above, and, accordingly, this rejection may be withdrawn.

Rejection of claims under 35 U.S.C. § 112, first paragraph (lack of utility)

All claims are rejected under 35 U.S.C. § 112, first paragraph, because one of skill in the art would not know how to use an invention with no apparent utility.

The Applicants respectfully submit that this rejection has been adequately addressed by the general discussion above, and, accordingly, this rejection may be withdrawn.

Rejection of claims under 35 U.S.C. § 112, first paragraph (enablement)

The claims are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which is not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Without wishing to acquiesce to the correctness of this rejection, the claims have been amended to recite polypeptides that are have 95% sequence identity to SEQ ID NO:8 *and can activate a p53 binding site controlled promoter when introduced into a mammalian cell*. The Applicants respectfully submit that in view the amendment and the above general discussion, this rejection has been adequately addressed and may be withdrawn.

Rejection of claims under 35 U.S.C. § 112, first paragraph (written description)

Claim 27 is rejected for reciting matter that is unsupported by the instant specification.

Without wishing to acquiesce to the correctness of this rejection, the claims have been amended to recite polypeptides that are have 95% sequence identity to SEQ ID NO:8 *and can activate a p53*

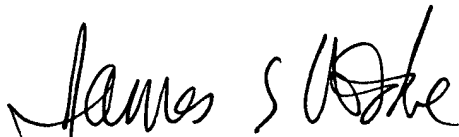
USSN: 09/715,725

binding site controlled promoter when introduced into a mammalian cell. The Applicants respectfully submit that in view the amendment and the above general discussion, this rejection has been adequately addressed and may be withdrawn.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number RIGL-008CIP.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: May 26, 2004

By: 
James S. Keddie, Ph.D.
Registration No. 48,920

BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

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09/715,725 Exhibit A



The Genetic Code

1st position (5' end)	U	C	A	G	3rd Position (3' end)
↓					↓
U	Phe Phe Leu Leu	Ser Ser Ser Ser	Tyr Tyr STOP STOP	Cys Cys STOP Trp	U C A G
C	Leu Leu Leu Leu	Pro Pro Pro Pro	His His Gln Gln	Arg Arg Arg Arg	U C A G
A	Ile Ile Ile Met	Thr Thr Thr Thr	Asn Asn Lys Lys	Ser Ser Arg Arg	U C A G
G	Val Val Val Val	Ala Ala Ala Ala	Asp Asp Glu Glu	Gly Gly Gly Gly	U C A G

Amino Acids Codons

A	Ala	Alanine	GCA GCC GCG GCU
C	Cys	Cysteine	UGC UGU
D	Asp	Aspartic acid	GAC GAU
E	Glu	Glutamic acid	GAA GAG
F	Phe	Phenylalanine	UUC UUU
G	Gly	Glycine	GGA GGC GGG GGU
H	His	Histidine	CAC CAU
I	Ile	Isoleucine	AUA AUC AUU
K	Lys	Lysine	AAA AAG
L	Leu	Leucine	UUA UUG CUA CUC CUG CUU
M	Met	Methionine	AUG
N	Asn	Asparagine	AAC AAU
P	Pro	Proline	CCA CCC CCG CCU
Q	Gln	Glutamine	CAA CAG
R	Arg	Arginine	AGA AGG CGA CGC CGG CGU
S	Ser	Serine	AGC AGU UCA UCC UCG UCU
T	Thr	Threonine	ACA ACC ACG ACU
V	Val	Valine	GUA GUC GUG GUU
W	Trp	Tryptophan	UGG
Y	Tyr	Tyrosine	UAC UAU

09/12, 25
EXHIBIT B

Sequence alignment of SEQ ID NO:8 and SEQ ID NO:2

```
SEQ ID NO:8  MGARVTPQDSGGLIGIENLPCELQRNFQLMRELDQRTEDKKAEIDILAAEYISTVKTLS  
:                                                    :  
SEQ ID NO:2  MRELDQRTEDKKAEIDILAAEYISTVKTLS  
  
SEQ ID NO:8  DQERVERQQKIQNAYSKCKEYSDDKVQLAMQTYEMVDKHIRRLDADLARFEADLKDKMEGS  
:           :  
SEQ ID NO:2  DQERVERLQKIQNAYSKCKEYSDDKVQLAMQTYEMVDKHIRRLDADLARFEADLKDKMEGS  
  
SEQ ID NO:8  DFESSGGRGLKKGRGQKEKRGSRGRGRRTSEEDTPKKKKHKGGSEFTDTILSVHPSDVLD  
:           :  
SEQ ID NO:2  DFESSGGRGLKKGRGQKEKRGSRGRGRRTSEEDTPKKKKHKGGSEFTDTILSVHPSDVLD  
  
SEQ ID NO:8  MPVDPNEPTYCLCHQVSYGEMIGCDNPDCEIWFHFACVDLTTPKPGKWFCPRCVQEKRK  
:           :  
SEQ ID NO:2  MPVDPNEPTYCLCHQVSYGEMIGCDNPDCEIWFHFACVDLTTPKPGKWFCPRCVQEKRK  
  
SEQ ID NO:8  KK  
:           :  
SEQ ID NO:2  KK
```

Sequence alignment of SEQ ID NO:8 and SEQ ID NO:4

```
SEQ ID NO:8  MGARVTPQDSGGLIGIENLPCELQRNFQLMRELDQRTEDKKAEIDILAAEYISTVKTLS  
:           :  
SEQ ID NO:4  MGARVTPQDSGGLIGIENLPCELQRNFQLMRELDQRTEDKKAEIDILAAEYISTVKTLS  
  
SEQ ID NO:8  DQERVERQQKIQNAYSKCKEYSDDKVQLAMQTYEMVDKHIRRLDADLARFEADLKDKMEGS  
:           :  
SEQ ID NO:4  DQERVERLQKIQNAYSKCKEYSDDKVQLAMQTYEMVDKHIRRLDADLARFEADLKDKMEGS  
  
SEQ ID NO:8  DFESSGGRGLKKGRGQKEKRGSRGRGRRTSEEDTPKKKKHKGGSEFTDTILSVHPSDVLD  
:           :  
SEQ ID NO:4  DFESSGGRGLKKGRGQKEKRGSRGRGRRTSEEDTPKKKKHKGGSEFTDTILSVHPSDVLD  
  
SEQ ID NO:8  MPVDPNEPTYCLCHQVSYGEMIGCDNPDCEIWFHFACVDLTTPKPGKWFCPRCVQEKRK  
:           :  
SEQ ID NO:4  MPVDPNEPTYCLCHQVSYGEMIGCDNPDCEIWFHFACVDLTTPKPGKW  
  
SEQ ID NO:8  KK
```

Exhibit C 09/715,725

p29ING4 and p28ING5 Bind to p53 and p300, and Enhance p53 Activity

Masayuki Shiseki, Makoto Nagashima, Remy M. Pedeux, Mariko Kitahama-Shiseki, Koh Miura, Shu Okamura, Hitoshi Onogi, Yuichiro Higashimoto, Ettore Appella, Jun Yokota, and Curtis C. Harris¹

Laboratories of Human Carcinogenesis [M. S., M. N., R. M. P., M. K-S., K. M., S. O., H. O., C. C. H.] and Cell Biology [Y. H., E. A.], Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland 20892-4255, and Biology Division, National Cancer Center Research Institute, Tokyo 104, Japan [J. Y.]

Abstract

We identified and characterized two new ING family genes, *p29ING4* and *p28ING5*, coding for two proteins of 249 and 240 amino acids, respectively. Both *p29ING4* and *p28ING5* proteins have a plant homeodomain finger motif also found in other ING proteins, and which is common in proteins involved in chromatin remodeling. *p29ING4* or *p28ING5* overexpression resulted in a diminished colony-forming efficiency, a decreased cell population in S phase, and the induction of apoptosis in a p53-dependent manner. Both *p29ING4* and *p28ING5* activate the *p21/waf1* promoter, and induce *p21/WAF1* expression. *p29ING4* and *p28ING5* enhance p53 acetylation at Lys-382 residues, and physically interact with p300, a member of histone acetyl transferase complexes, and p53 *in vivo*. These results indicate that *p29ING4* and *p28ING5* may be significant modulators of p53 function.

Introduction

A candidate tumor suppressor gene, *ING1*, was cloned by the genetic suppressor element methodology (Ref. 1; reviewed in Ref. 2). The *ING1* gene is located at chromosome 13q33–34, where allelic losses are observed frequently in several types of human cancers (2). Initial studies demonstrated that the originally cloned *ING1* gene product, p33ING1, is a negative growth regulator, which plays a role in senescence and apoptosis (3, 4), and physically associates with the p53 tumor suppressor protein (5). The *p53* gene is inactivated frequently in many types of human cancers (6–8), and p53-mediated cellular processes play key roles in human carcinogenesis (9). The *ING1* gene encodes three different isoforms, p47ING1a, p33ING1b, and p27INGc, by alternative splicing (10, 11). All three of the isoforms share a PHD²-finger motif in their COOH-terminal ends, which are found in proteins involved in chromatin remodeling, suggesting they are acting as transcription regulators (12). This hypothesis is supported by studies showing that three yeast proteins, Yng1, Yng2, and pho23, which have a PHD-finger motif at their COOH-terminal regions and have homology with human p33ING1, are associated with HAT complexes (13–15). Additional studies indicate that three isoforms of the *ING1* differentially associated with HDAC complexes or HAT complexes (16–18).

We cloned and characterized the *p33ING2* gene recently, which shows high homology to *p33ING1* (19). *p33ING2* suppressed cell growth by induction of the G₁/S arrest and apoptosis in a p53-dependent manner. In addition, *p33ING2* may modulate p53 function through p53 acetylation, and play a role in DNA double-strand break repair. Computational searching indicated the existence of three ad-

ditional ING family genes. We and others identified and characterized a third member of the family, *p47ING3* (20, 21). In the present study, two new ING family genes, *p29ING4* and *p28ING5*, have been identified, and the potential mechanism of their interactive effects with p53 has been investigated.

Materials and Methods

cDNA Cloning. A computational search was performed to find human expressed sequence tag clones showing high homology with *p33ING1b* and *p33ING2* cDNAs by using a BLAST program (National Center for Biotechnology Information). To determine the entire cDNA sequence, the rapid amplification cDNA end method was carried out with the human placenta Marathon-Ready cDNA (Clontech).

Cell Lines, Expression Vectors, and Transfection. A human colorectal cancer cell line, RKO, and its isogenic subclone, RKO-E6, with p53 inactivated by ubiquitin-dependent cleavage mediated by the E6 protein of human papilloma virus (22), were used for the colony formation efficiency assay, cell cycle analysis, apoptotic assay, and analysis for p53 post-translational modification. In addition, two cell lines, AsPC-1 (pancreatic cancer cells having a truncated p53; Ref. 23) and OsA-CL (osteosarcoma cells having a wild-type p53) were used for the colony formation assay.

The coding regions of *p29ING4* and *p28ING5* cDNAs were isolated from human placenta Marathon-Ready cDNA (Clontech) by PCR. After digesting with the appropriate restriction enzyme, cDNAs were cloned into pcDNA3.1 (+)-Neo or pcDNA3.1 (+)-Hgr expression vectors (Invitrogen), producing the pcDNA3.1-ING4 and pcDNA3.1-ING5 vectors. The cDNA was also cloned into pFLAG-CMV-2 mammalian expression vectors (Sigma, St. Louis, MO) to generate pFLAG-ING4 and pFLAG-ING5 vectors, producing NH₂-terminal FLAG fusion p29ING4 and p28ING5 proteins in mammalian cells. The pcDNA3.1-ING1b and pcDNA3.1-ING2 expression vectors that we described previously (19) were also used in some experiments. In the present study, cells were transfected with a Lipofectamine Plus reagent (Invitrogen) according to the manufacture's instructions. The amounts of the reagents were adjusted with the scale of the experiments.

Antibodies. Rabbit polyclonal antibodies against p29ING4 and p28ING5 were generated by the injection of chemically synthesized keyhole limpet hemocyanin-conjugated oligopeptides, of which the sequence corresponds to amino acids 121–129 in the p29ING4 protein and 57–65 in the p28ING5 protein, respectively. The antisera from immunized rabbits were purified with the oligopeptides coupled with SulfoLink (Pierce). Homology between p29ING4 and p28ING5 is high, but it was checked that antibodies do not cross-react. Anti-p53 (DO-1; Calbiochem), antiacetylated p53 antibody (Calbiochem), antiphospho-p53-specific antibodies (Cell Signaling Technology), anti-WAF1 (Calbiochem), anti-BAX (Ab-1; Santa-Cruz Biotechnology), anti-p300 (C-20 or N-15; Santa-Cruz Biotechnology), and mouse monoclonal anti-FLAG M2 antibody (Sigma) were also used for Western blot analysis or immunoprecipitation analysis.

Colony Formation Assay. Four cancer cell lines, RKO, RKO-E6, AsPC-1, and OsA-CL, were used for the experiments. Cells were plated on six-well plates (2×10^4 cells/cm²), cultured for 24 h at 37°C, and then transfected with 1 µg of pcDNA3.1-ING4, pcDNA3.1-ING5, or pcDNA3.1 (control). Cotransfection experiments using the pC53-SN vector containing wild-type p53 (24) were carried out for AsPC-1 cells. For RKO and RKO-E6 cells, the expression vectors containing hygromycin-resistant gene were used, and cells were cultured in selection medium containing 200 µg/ml of hygromycin (Sigma). For AsPC-1 and OsA-CL cells, the expression vectors containing neomycin-

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¹ To whom requests for reprints should be addressed, at Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, NIH, 37 Convent Drive, Building 37, Room 2C05, Bethesda, MD 20892-4255. Phone: (301) 496-2048; Fax: (301) 496-0497; E-mail: Curtis_Harris@nih.gov.

² The abbreviations used are: PHD, plant homeodomain; HAT, histone acetyl transferase; HDAC, histone deacetylase; PI, propidium iodide; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling.

resistant genes were used for transfections, and cells were cultured in the selection medium containing 800 $\mu\text{g}/\text{ml}$ of neomycin (Sigma). After 2-week selection, cells were fixed on the plates with formaldehyde (10%) and stained with crystal violet. Then, colonies were counted. The data are shown as the average with SD of three independent experiments. Each experiment was performed in triplicate.

Cell Cycle Analysis. RKO or RKO-E6 cells were plated on 60-mm dishes at 2×10^4 cells/ cm^2 24 h before the transfection. Cells were cotransfected with 2 μg of pcDNA3.1-ING4, pcDNA3.1-ING5, or pcDNA3.1 (control), and with 200 ng of pEGFP-F vector (Clontech) as a marker for transfection. Forty-eight h after transfection, cells were harvested, washed with PBS, and then fixed with 70% ethanol for >3 h. After ethanol was removed by centrifugation, pellets were resuspended with a PI-RNase solution, containing 20 $\mu\text{g}/\text{ml}$ of PI (Sigma) and 100 $\mu\text{g}/\text{ml}$ of RNase A (Sigma), and incubated for 30 min at room temperature. DNA content of the cells was measured with a FACSCalibur flow cytometer (Becton-Dickinson). Cell cycle profiles in diploid cells were analyzed using the MODFIT LT Program (Verity Software House). At least 10,000 of the GFP-positive cells, which are considered to be transfection-positive cells, were analyzed.

Detection of Apoptosis. RKO or RKO-E6 cells were plated onto six-well chamber slides at 1×10^4 cells/ cm^2 and cultured for 24 h. Cells were cotransfected with 50 ng of pcDNA3.1-ING4, pcDNA3.1-ING5, or pcDNA3.1 (control) vectors, and 5 ng of the pEGFP-F vector as a marker for transfection efficiency. Cells were fixed with 4% paraformaldehyde 24 h after transfection, and apoptotic cells were detected using the TUNEL method. Fragmented DNA was labeled by terminal transferase (Boehringer Mannheim) with AlexaFluor 568-5-dUTP (Molecular Probes). Cells were stained by 4',6-diamidino-2-

phenylindole (Vector Laboratories), and apoptotic cells were counted in GFP populations by fluorescent microscopy.

Detection of Post-Translational Modifications of p53. RKO cells were seeded in a 100-mm dish at $2 \times 10^4/\text{cm}^2$ and cultured for 24 h. Cells were transfected with pcDNA3.1-ING1b, pcDNA3.1-ING2, pcDNA3.1-ING4, pcDNA3.1-ING5, or pcDNA3.1 (control) vectors. The pcDNA3.1-ING1b and pcDNA3.1-ING2 vectors were used in our previous studies (19, 20). Cells were harvested 24 h after transfection. To detect p53 acetylation, cells were treated for 3 h with trichostatin A (Wako; 5 μM) before they were harvested. To detect acetylated p53, immunoprecipitation was performed with agarose-conjugated anti-p53 antibodies, DO-1 (Calbiochem) and Pab 240 (Santa Cruz Biotechnology). Samples were analyzed by Western blot to detect the acetylated p53 at Lys-382 residue. For detection of phosphorylated p53 at Ser-15 or Ser-392 residues, whole cell lysates were used.

Immunoprecipitation. Whole cell lysates were prepared from transfected RKO cells using an ice-cold lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), and 0.5% NP40, with complete protease inhibitor mixture (Roche Molecular Biochemicals). For immunoprecipitation, agarose-conjugated antibodies were used: rabbit polyclonal anti-p300 (N-15; Santa Cruz Biotechnology), mouse monoclonal anti-FLAG M2 antibody (Sigma), and anti-p53 antibodies, DO-1 and Pab240. Two μg of the antibody were incubated with 1 mg of the lysate for 1 h. In some experiments, to block specific binding between antibody and antigen, a specific-blocking peptide (20 μg) was mixed with the antibody and incubated for 2 h at room temperature, before adding the lysate. After washing, the samples were analyzed by Western blot to detect p53 and other proteins.

p33ING1b	1	-----MLSPANGEQLHLVN-VVEDYLDSTESLPDLORNVSIMREIDAKYQEL
p33ING2	1	MLGGOOQOQLYSSAALLTGRSRLTCYVODYLECVBSLEPHDMORNVSIMREIDAKYQETL
p47ING3	1	-----MLYLEDYLEMTBOLPMDLRDRFETEMREMDLQVQNAM
p29ING4	1	-----MAAGMYLEHYLDSTENLPDLORNFMRLMDLQRTEDLK
p28ING5	1	-----KATAMYLEHYLDSTENLPDLORNFMRLMDLQRTEDRK
p33ING1b	49	KELDECYERFS---RETDCAQKRMTHCVORALIRSOELGDEKIQIVSQVVELVENRTRQ
p33ING2	61	KETIDVYERFK---KEDDLNQKKRLQQLQALINSQELGDEKIQIVTQMLELVENRARRQ
p47ING3	37	DOLEQRVSEEFMNAKKNKPEWRECOMASIKKDYKALEDADEKVLQANQYDLDVDRHLRK
p29ING4	39	AEIDKLATEYMSARSLSSEELKALLKQIQSAYGCKEFGDDKVOLAMQIYEMVDKHIRR
p28ING5	39	AEIDILAAEYISTVKTLSPEQVERLQKIQNAYSCKEYSDDKVOLAMQIYEMVDKHIRR
p33ING1b	106	VDSHVELFEAQELG-----
p33ING2	118	MELHSQCFQDPAES-----
p47ING3	97	LDQELAKEMLEADNAGITEILERRSLELDTPSQPVNNHHAHSNTEVEKRRKYNPTSHHT
p29ING4	100	LDTDLARFEADLKEK-----
p28ING5	100	LDADLARFEADLKOK-----
p33ING1b	121	-----DTAGNSGKAGADREKGEAAAQAD
p33ING2	131	-----ERASDKAKMDSQPEERSSR
p47ING3	157	TIDHIPEKKPKSEALLSTLTSDASKENTLGCRRNNSTASSNNAYNVNSSLGSGYNIGSL
p29ING4	115	-----QIESDDYDSSSSKKGK-----
p28ING5	115	-----MEGSDFESSGGRGK-----
p33ING1b	144	KPNS-----KRSRRQNNENRENASSNHDH-----
p33ING2	151	RP-----RRORTSESRDLCHMANGI-----
p47ING3	217	SSGTGAGAITMAAAQAVQATAQMKEGRRTSSLKASYEAFKNNDFQLGKEFSMARETVGYS
p29ING4	130	-----KKGRTQKEKKAARARSKG-----
p28ING5	129	-----KKGRCQKEKGRSGRGR-----
p33ING1b	169	-----DDGASGTPEK-----EKAK-----TSKKK-----
p33ING2	171	-----EDCDDOPPEK-----EKSK-----SAKKK-----
p47ING3	277	SSSALMTTLTQNASSSAADSRSGRKSKNNKSSSQSSSSSSSSSSSSSSSSSTTVVQEIS
p29ING4	148	-----KNSDEAEPT-AOKKLLKV-RTSPETG-----
p28ING5	145	-----RTSEEDTPEK-----KKHK-----CGSEF-----
p33ING1b	188	-KRSKAKAEREASPALEIDPNEPTYCLCNQVSYGEMIGCDNDECPIEWFHEFCVGLNNHK
p33ING2	190	-KRSKAKQEREASPEFAIDPNEPTYCLCNQVSYGEMIGCDNEOCPIEWFHEFCVSLTYK
p47ING3	337	QQTTVVPESDSNSQVDWTYDPNEPRYICNQVSYGEMVGGCDNDCPIEWPHYGCVGLTEA
p29ING4	173	MPSVTFGSVHPSDVLEMDVDPNEPTYCLCHOVSYGEMIGCDNDCPIEWFHEFCVGLTTK
p28ING5	164	--TDTILSVHPSDVLEMDVDPNEPTYCLCHOVSYGEMIGCDNDCPIEWFHEFCVDLTTK
p33ING1b	247	PKGKMYCPKCRGNEKTMDKALEKSKKERAYNR
p33ING2	249	PKGKMYCPKCRGDNKTMDKSTETKTKDRSR-
p47ING3	397	PKGKMYCPQCTAAMKRRGSRHK-----
p29ING4	233	PKGKMYCPKCRGSEKRRK-----
p28ING5	223	PKGKMYCPKCRGSEKRRK-----

Fig. 1. Comparison of five ING family protein sequences. * below sequence alignments indicate a PHD-finger motif, Cys (4)-His-Cys (3).

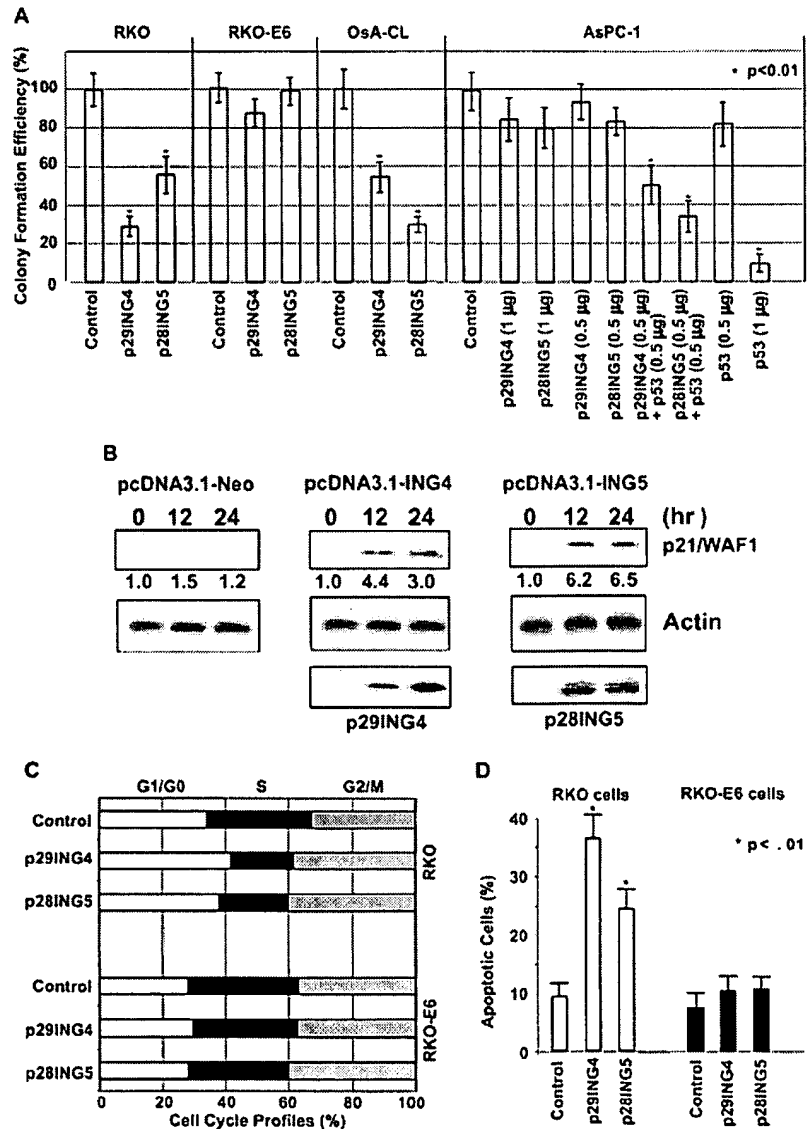
Results

cDNA Cloning of the *p29ING4* and *p28ING5* Genes. We found several human EST clones showing a high homology with the *p33ING1b* and *p33ING2* cDNAs by a BLAST computational search. By aligning those clones, we found two new ING family genes, *p29ING4* and *p28ING5*. An additional computational search and rapid amplification cDNA end method were carried out to determine the entire cDNA sequences. *p29ING4* and *p28ING5* cDNAs consist of 1380 and 1068 nucleotides, and encode 249 and 240 amino acids, respectively. The entire nucleotide sequences of the *p29ING4* and *p28ING5* genes were deposited in GenBank, and the accession numbers are AF 156552 for *p29ING4*, and AF 189286 for *p28ING5*. *p29ING4* and *p28ING5* proteins show high homology to other ING family proteins in their NH₂-terminal and COOH-terminal ends (Fig. 1). *p29ING4* and *p28ING5* show especially high homology with each other (similarity, 80.3%; identity, 72.8%). Similar to other ING family proteins, *p29ING4* and *p28ING5* have a PHD-finger motif in their COOH-terminal region as well as two nuclear localization sequences.

***p29ING4* and *p28ING5* Negatively Control Cell Growth in a p53-dependent Manner.** We investigated the effect of exogenous *p29ING4* and *p28ING5* overexpression on cell growth, and its p53

dependency, using RKO cells and its isogenic subclone, RKO-E6 cells, in which p53 is inactivated by the human papilloma viral oncoprotein E6 (22). *p29ING4* or *p28ING5* overexpression significantly reduced the colony formation of RKO cells (Fig. 2A), whereas *p28ING5* overexpression showed no effect and *p29ING4* overexpression showed minimal effects on the colony formation of RKO-E6 cells. *p29ING4* or *p28ING5* overexpression also resulted in a statistically significant reduction of the colony formation of the OsA-CL cells, which contain wild-type p53. In contrast, *p29ING4* or *p28ING5* overexpression showed a minimal effect on the colony formation efficiency of the AsPC-1 cells containing mutant p53. Transfection with 1 μ g of pC53-SN markedly reduced the colony formation of the AsPC-1 cells; however, transfection with 0.5 μ g of the vector was insufficient for suppression of colony formation of the AsPC-1 cells. Cotransfection of pcDNA3.1-ING4 or pcDNA3.1-ING5 (0.5 μ g) and p53 (0.5 μ g) resulted in a significant reduction of the colony formation of AsPC-1 cells. *p29ING4* or *p28ING5* modulate the transcriptional activity of p53 as the activity of the *p21/waf1* promoter (a p53-regulated gene) showed a modest but significant increase when *p29ING4* or *p28ING5* were overexpressed in RKO cells (data not shown). This was not the case in RKO-E6 cells (data not shown). To

Fig. 2. Negative regulation of cell proliferation by *p29ING4* and *p28ING5*. **A**, colony formation assay. RKO or RKO-E6 cells were seeded in six-well plates, and transfected with pcDNA3.1-ING4, *p28ING5*, or pcDNA3.1 (control) vectors, which have a hygromycin-resistant gene. After 2 weeks of hygromycin selection (200 μ g/ml), cells were fixed and stained by crystal violet for colony counting. OsA-CL and AsPC-1 were seeded in six-well plates, and transfected with pcDNA3.1-ING4, pcDNA3.1-ING5, or pcDNA3.1 (control) vectors, which have a G418-resistant gene. After 2 weeks of G418 selection (800 μ g/ml), cells were fixed and stained by crystal violet for colony counting. Data are shown as relative values to the control, and represent the average of three independent experiments; bars, \pm SD. Statistical analysis was carried out using Student's *t* test. **B**, increased expression of the *p21/waf1* protein by *p29ING4* or *p28ING5* overexpression. RKO cells were transfected with pcDNA3.1 (control), pcDNA3.1-ING4, or pcDNA3.1-ING5 vectors. Cells were harvested at different time points after transfection. Whole cell lysates were extracted, 15 μ g of each lysate was electrophoresed, and then subjected to Western blot analysis to detect *p21/WAF1* and *p29ING4* or *p28ING5*. Numbers below the bands are densitometry values as a relative ratio to the control. **C**, effect of the overexpression of *p29ING4* and *p28ING5* on cell cycle profiles. RKO or RKO-E6 cells were cotransfected with pcDNA3.1-ING4, pcDNA3.1-ING5, or pcDNA3.1 (control), and pEGFP-F vector (Clontech) with Lipofectamine Plus Reagent (Invitrogen). Forty-eight h after transfection, cells were fixed and stained with PI, and DNA content of the cells was measured by flow cytometry (FACSCalibur; Becton-Dickinson). Cell cycle profiles were analyzed using MODFIT LT Program (Verity Software House). At least 10,000 of the GFP-positive cells were analyzed. **D**, induction of apoptosis. RKO or RKO-E6 cells were plated onto eight-well chamber slides, and cotransfected with the pcDNA3.1-ING4, pcDNA3.1-ING5, pcDNA3.1 (control), or pEGFP-F vectors. Cells were fixed 24 h after transfection, and fragmented DNA was detected by TUNEL assay. The slides were observed by fluorescent microscopy, and 100 GFP-positive cells were analyzed for apoptosis. Data are shown as a percentage and represent the average of three independent experiments; bars, \pm SD. Statistical analysis was carried out by Student's *t* test.



confirm these results, we performed Western blot analysis to examine whether p21/WAF1 expression was induced by either p29ING4 or p28ING5 overexpression (Fig. 2B). The expression of p21/WAF1 was markedly increased by p29ING4 or p28ING5 overexpression in RKO cells, although minimal induction of the p21/WAF1 by control vector was observed. In RKO-E6 cells, no p21/WAF1 increase was observed when p29ING4 or p28ING5 were overexpressed (data not shown). Cell cycle analysis indicated that p29ING4 and p28ING5 overexpression result in a decreased S phase population, and increased G₁/S and G₂/M phases 48 h after transfection of RKO cells, but no significant cell cycle change was seen in RKO-E6 cells (Fig. 2C). The TUNEL assay showed that a significantly higher number of RKO cells transfected with pcDNA3.1-ING4 or pcDNA3.1-ING5 underwent apoptosis when compared with RKO cells transfected with pcDNA3.1 (control; Fig. 2D). No apoptotic induction was observed in RKO-E6 cells. The percentage of the apoptotic cells in RKO or RKO-E6 cells transfected with p29ING4 or p28ING5 was also quantified as a sub-G₁ population by flow cytometry (data not shown), and the results were similar.

p29ING4 and p28ING5 Increase p53 Acetylation at Lys-382 Residue and Physically Interact with p300, a Component of HAT Complexes. To determine the mechanism by which p29ING4 and p28ING5 modulate p53 function, we investigated whether either p29ING4 or p28ING5 are involved in mediating p53 post-translational modifications, which are thought to be the major mechanisms modulating its functions. The acetylation and phosphorylation status of the Lys-382, Ser-15, or Ser-392 residues in p53 were examined by Western blot analysis, using antiphosphorylated p53 or antiacetylated p53-specific antibodies. As we reported previously, p33ING2 increased p53 Lys-382 acetylation, whereas p33ING1b did not (11979). p29ING4, and to a lesser extent, p28ING5, increased p53 Lys-382 acetylation (Fig. 3A). p33ING2, p29ING4, and p28ING5 increased the amount of acetylated p53 at Lys-382 residue when compared with pcDNA3.1 (control). Expression of these proteins did not change the amount of p53 protein detected by the anti-p53 antibody (DO-1), indicating that p53 acetylation at Lys-382 residue induced by p33ING2, p29ING4, or p28ING5 was not associated with an increased amount of the p53 protein. The p53 phosphorylation levels at the Ser-15 and Ser-392 residues were not changed by p29ING4 or p28ING5 (data not shown), or p33ING1b, p33ING2, or p47ING3 protein overexpression (Refs. 18, 19; data not shown). *In vivo* physical interactions between p29ING4 or p28ING5 and p300 were examined by coimmunoprecipitation. RKO cells, transfected with either FLAG-ING4 or FLAG-ING5, were immunoprecipitated with either the anti-FLAG (Fig. 3B) or anti-p300 antibody (Fig. 3C), and anti-p300 or anti-FLAG antibodies were used for detection by Western blot. Several bands may be detected for p300 reflecting a post-translationally modified protein (25) or a nonspecific band (Fig. 3B). In all of the experiments, the presence of either p300 or FLAG was detected, indicating that ING4 and ING5 physically interact with p300 (Fig. 3C). Therefore, p29ING4 and p28ING5 are likely to be present in certain HAT complexes.

Interaction between p53 and p29ING4 or p28ING5 *in Vivo*. Previous studies indicated that p33ING1b physically interacts with p53, but p33ING2 and p47ING3 do not (5, 18, 19). In the present study, we investigated whether p29ING4 or p28ING5 binds to p53 *in vivo*. p53 was coimmunoprecipitated with p29ING4 with anti-ING4 antibody, but not with preimmunized rabbit IgG, and specific-blocking peptide inhibited the coimmunoprecipitation (Fig. 4A). p53 was coimmunoprecipitated with the anti-ING5 antibody in a cell lysate of RKO cells, but not in immunoprecipitations with preimmunized rabbit IgG. A blocking peptide inhibits p53 coimmunoprecipitation with anti-ING5 antibody (Fig. 4B). In reverse experiments, cell extracts

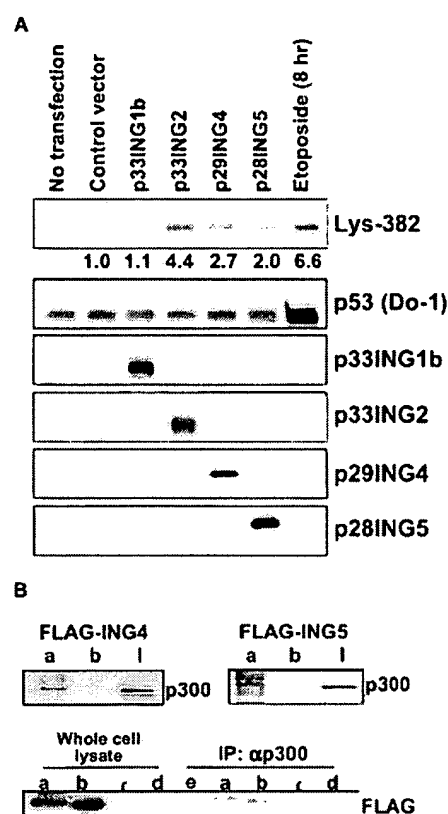


Fig. 3. A, post-translational modifications of p53 by ING family overexpression. RKO cells were transfected with pcDNA3.1-ING1b, pcDNA3.1-ING2, pcDNA3.1-ING4, pcDNA3.1-ING5, or pcDNA3.1 (control). Acetylation of p53 at Lys-382 was detected in anti-p53 (DO-1 and Pab240) immunoprecipitates from RKO cells by Western blot analysis. Total p53 was detected with DO-1 antibody. ING proteins were detected with specific antibody. RKO cells treated with etoposide (20 μ M/ml) for 8 h were used as a positive control for acetylated p53. Numbers below the bands are densitometry values as a relative ratio to the control. B and C, *in vivo* interactions between p29ING4 or p28ING5 and p300. B, coimmunoprecipitations of p300 and p29ING4 or p28ING5. Cell lysates (1 mg) from the RKO cells transfected with FLAG-ING4 or FLAG-ING5 were immunoprecipitated with anti-FLAG M2 antibody alone (a) or with FLAG peptide (b). Immunoprecipitates were analyzed with Western blot to detect p300. Whole cell lysates (20%) were also electrophoresed together on the gel (f). C, coimmunoprecipitations of p29ING4 or p28ING5 with p300. Cell lysates from RKO cells transfected with FLAG-ING4 or FLAG-ING5 were immunoprecipitated with anti-p300 (N-15). (a) RKO transfected with FLAG-ING4, (b) RKO transfected with FLAG-ING5, (c) RKO transfected with empty FLAG vector, (d) RKO lysate, and (e) no lysate. Immunoprecipitates were analyzed with Western blot to detect p29ING4 or p28ING5 with FLAG antibody.

from RKO cells transfected with FLAG-ING4 or FLAG-ING5 were immunoprecipitated with anti-p53 murine monoclonal antibodies (DO-1 and Pab240). p29ING4 and p28ING5 were clearly detected in anti-p53 antibody immunoprecipitation (Fig. 4C). Whole cell lysates used are the same as for Fig. 3C. Therefore, p29ING4 and p28ING5 physically interact with p53 *in vivo*.

Discussion

ING genes have been reported to be implicated in apoptosis, cell cycle regulation, and DNA repair. They are rarely mutated in human cancer, but expression is down-regulated in several tumor types (2). We identified two new ING family genes, p29ING4 and p28ING5. As for all of the ING proteins, p29ING4 and p28ING5 have a PHD-finger motif in their COOH-terminal regions and nuclear localization sequences. The PHD-finger motif is found frequently in proteins, which are transcriptional regulators and involved in chromatin remodeling (12). Overexpression of either p29ING4 or p28ING5 in cancer cells resulted in reduced colony formation efficiency, decreased S phase population, and increased apoptosis. These effects were p53-dependent, indicating that those two proteins are potential regulators of

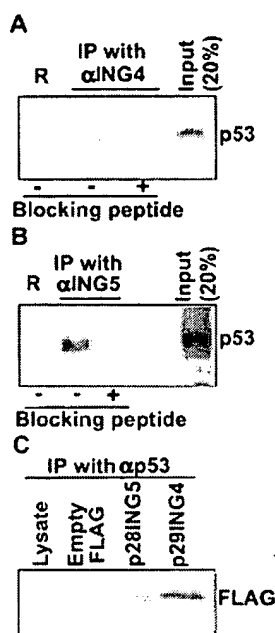


Fig. 4. *In vivo* interaction between p29ING4 or p28ING5 and p53. *A*, *in vivo* interaction between p29ING4 and p53. Cell lysates extracted from RKO cells transfected with pcDNA3.1-ING4 were immunoprecipitated with rabbit preimmunized IgG (R), or anti-ING4 antibody with or without specific-blocking peptide. *B*, *in vivo* interaction between p28ING5 and p53. p53 was detected by DO-1 antibody. Cell lysates from RKO cells transfected with pcDNA3.1-ING5 were immunoprecipitated with preimmunized rabbit IgG (R), or anti-ING5 antibody with or without blocking peptides. *C*, cell lysates extracted from RKO cells, transfected with FLAG-ING4 or FLAG-ING5, were immunoprecipitated with anti-p53 antibodies (DO-1 and Pab 240), and p29ING4 and p28ING5 were detected by Western blot analysis using anti-FLAG antibody. Whole cell lysates used are also the same for Fig. 3C.

p53-mediated cellular processes, and may function as tumor suppressors, as proposed for other ING family proteins identified and characterized previously (1–5, 19, 20). Previous studies showed that p33ING1, p33ING2, and p47ING3 modulate p53 transcriptional activity. Although p53 is a multifunctional protein, its primary function is transcriptional activation. To better understand the mechanism(s) by which p28ING4 and p28ING5 are involved in p53-mediated cellular pathways, we initially investigated the effect of the p29ING4 and p28ING5 on a p53-responsive promoter. *p21/waf-1* is a well-characterized p53-regulated gene of which the promoter contains consensus sequences of the p53-binding sites (26). Our results show that p29ING4 or p28ING5 overexpression activates the *p21/waf-1* promoter, and increases p21/WAF1 protein in RKO cells, but not in RKO-E6 cells. Thus, p29ING4 and p28ING5 can modulate p53 transcriptional activity. The modest activation of the *p21/waf-1* promoter by p29ING4 and p28ING5 most likely reflects the lack of increase in total endogenous p53 level. p53 functions as a transcriptional transactivator and may be regulated by interacting with other proteins or post-translational modifications (27). p29ING4 and p28ING5 physically interact with p53 (Fig. 4) and, therefore, could regulate p53 by recruiting cofactors. Mainly, our results suggest that p29ING4 and, to a lesser extent, p28ING5 enhance acetylation of p53 on K382, indicating that these two proteins could modulate p53 function through the control of its acetylation status. p53 phosphorylation at serine 15 and 392 was not changed by p29ING4 or p28ING5 overexpression. However, it cannot be excluded that other phosphorylation or acetylation sites may be affected.

ING proteins are conserved through the evolution, and yeast homologues, Yng1 and Yng2, have been found to be part of HAT and/or HDAC complexes (13–15). It has been found recently that HATs and HDACs control acetylation of lysine residues not only in histone, but

also in other cellular proteins, including p53 (13–15). We found previously that p33ING2, but not p47ING3, induced acetylation on K382 (19, 20). In the present report, we show that either ING4 or ING5 can induce p53 acetylation on K382. Because ING family proteins do not show sequence similarities to the proteins having HAT or HDAC enzymatic activities, p29ING4 and p28ING5 are more likely to be subunits of HAT or HDAC complexes and, thus, modulate p53 acetylation, enhancing p53 transcriptional activity. Several HATs, among them p300 and PCAF, and HDACs, such as HDAC1 and hSir2, regulate acetylation of p53 (28–33). How do p29ING4 and p28ING5 increase p53 acetylation? Three isoforms of the ING1 proteins differently associate with HDACs or HATs (16–18). Our immunoprecipitation experiments indicate that p29ING4 and p28ING5 bind to p53, and also associate with p300, a component of HAT complexes. Similar results have been reported with p33ING1b (17, 18).

There are some uncertainties about the significance of p53 acetylation (34). *In vivo*, chromatin immunoprecipitation analyses failed to show that p53 acetylation changes p53 sequence-specific DNA binding (35–37). p53 acetylation most likely modulates p53 function through other mechanisms, including the recruitment of transcriptional coactivators such as p300/CBP and PCAF (35, 36), regulation of ubiquitylation (38–42), and changes in p53 subcellular localization (42, 43). More significant roles for p29ING4 and p28ING5 possibly exist in the modulation of p53 function, not only through p53 acetylation, but also the recruitment of coactivators including HAT complexes, or repressors including HDAC complexes, with p53 at p53-responsive promoter regions, leading to chromatin remodeling and the induction of transactivation of p53-responsive genes. Our results indicate that p29ING4 and p28ING5 modestly activate the exogenous *p21/waf-1* promoter, whereas the p21/WAF1 protein level is markedly increased by p29ING4 or p28ING5. Their stronger effects on an endogenous promoter with a chromatin structure, rather than exogenous artificial promoters without a chromatin structure, are consistent with a mechanism of chromatin remodeling.

There are at least seven ING family proteins, including three ING1 gene products and four other ING family gene products. The different biological and biochemical pathways in which these genes are involved are just beginning to be unraveled, but it is clear that they have different functions. A most interesting finding is their ability to modulate p53 activity, which may depend on their interactions with different HAT and HDAC proteins. Additional analyses, including a genome-wide search for the effect of overexpression of p29ING4 or p28ING5 on the expression profiles, are necessary to identify which set of the genes, including p53-responsive genes, are induced or suppressed by p29ING4 or p28ING5. The ING family proteins may also vary in their distribution and regulation in different cell types.

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EXHIBIT D

Sequence alignment of p28ING5 and SEQ ID NO:8

SEQ ID NO:8 MGARVTPQDSGGLIGIENLPCELQRNFQLMRELDQRTEDKKAIEDILAAEYISTVKTLSP
:
p28ING5 MATAMYLEHYLDSIENLPCELQRNFQLMRELDQRTEDKKAIEDILAAEYISTVKTLSP

SEQ ID NO:8 DQ RVERQQKIQNAYSKCKEYSDDKVQLAMQTYEMVDKHIRRLDADLARFEADLKDKMEGS
::: :
p28ING5 DQ RVERLQKIQNAYSKCKEYSDDKVQLAMQTYEMVDKHIRRLDADLARFEADLKDKMEGS

SEQ ID NO:8 DFESSGGRGLKKGRGQKEKGRSRRGRRTSEEDTPKKKKHKHKGSEFTDTILSVHPDSVLD
 ::
 p28ING5 DFESSGGRGLKKGRGQKEKGRSRRGRRTSEEDTPKKKKHKHKGSEFTDTILSVHPDSVLD

SEQ ID NO:8 MPVDPNEPTYCLCHQVSYGEMIGCDNPDCPIEWFHFACVDLTTKPKGKWFPCRCVQEKRK
 ::
 p28ING5 MPVDPNEPTYCLCHQVSYGEMIGCDNPDCPIEWFHFACVDLTTKPKGKWFPCRCVQEKRK

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SEQ ID NO:8  KK
              ::
p28ING5      KK

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